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Genetics

Green fluorescent protein as a vital marker and reporter of gene expression in *Drosophila*

(targeted expression/enhancer trap/GAL4/confocal microscopy)

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ABSTRACT **We have used the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* as a vital marker/reporter in *Drosophila melanogaster*. Transgenic flies were generated in which GFP was expressed under the transcriptional control of the yeast upstream activating sequence that is recognized by GAL4. These flies were crossed to several GAL4 enhancer trap lines, and expression of GFP was monitored in a variety of tissues during development using confocal microscopy. Here, we show that GFP could be detected in freshly dissected ovaries, imaginal discs, and the larval nervous system without prior fixation or the addition of substrates or antibodies. We also show that expression of GFP could be monitored in intact living embryos and larvae and in cultured egg chambers, allowing us to visualize dynamic changes in gene expression during real time.**

Development is the cumulative effect of dynamic changes in gene expression in different cells within an organism. At present, several techniques exist that allow an examination of gene expression through the measurement of either RNA or protein distribution within fixed tissue. Gene expression can be measured either directly by using probes and antibodies or indirectly by detecting the product of a fusion between the gene of interest and a reporter gene such as bacterial *lacZ* (1). In *Drosophila*, *lacZ* is often used in enhancer trap screens to identify genes that are expressed in a tissue-specific manner (2–4) or as a reporter to identify tissue-specific regulatory regions within known genes. All of these approaches are limited in that they only provide a static image of changes in gene expression during development. Furthermore, these techniques usually involve extensive manipulation including dissection of the tissue of interest, fixation, and the addition of various substrates or antibodies, and they are of limited use in living tissue.

Recently, Chalfie *et al.* (5) described the use of the green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* as a vital reporter for gene expression in both bacteria and *Caenorhabditis elegans*. In that study, GFP was placed under the transcriptional control of the *mec-7* promoter, which is activated in a small number of *C. elegans* neurons. GFP was nontoxic to cells, and its expression did not appear to interfere with cell growth and/or function. In addition, the green fluorescence did not appear to photobleach when viewed with fluorescein filter sets. These results suggested that GFP might be a powerful tool to examine changes in gene expression in living tissue.

Subsequently, GFP has been used in *Drosophila* to monitor the subcellular distribution of the exuperantia protein (exu) (6). In those studies, GFP was expressed as an in-frame fusion with the exu protein (encoded by *exu*) under the transcriptional control of its own promoter. The exu–GFP fusion protein was found to be expressed in the same pattern as the native exu protein. These results demonstrated the potential of GFP as a vital marker in *Drosophila*. However, the fact that GFP was produced as a exu–GFP fusion protein that was only expressed in adult ovaries precludes its use as a general marker/reporter gene. In theory, the exu–GFP fusion protein could be targeted to other tissues using various promoters, but the effects of ectopically expressing an exu–GFP fusion protein in other cell types are uncertain. Alternatively, additional fusion proteins could be generated with GFP, but this approach would be cumbersome, and the fusion products may be unstable, inactive, or nonfluorescent.

To make GFP more generally useful, we have utilized the GAL4 enhancer trap technique developed by Brand and Perrimon (7) to target expression of GFP. Here we show that GFP can be used as a vital marker of gene expression in a variety of living cell types at various developmental stages. We also demonstrate that GFP need not be expressed as a fusion protein in *Drosophila* but can be utilized directly as a reporter gene, much like *lacZ*. Finally, we show that GFP can be used to detect dynamic changes in gene expression in living tissue. Taken together, our results indicate that GFP will prove to be a powerful tool for viewing developmental changes within a living organism.

MATERIALS AND METHODS

Enhancer Detection Screen. GAL4-expressing enhancer trap lines were generated by mobilizing a single X chromosome-linked GAL4 *P*-element insertion (pGawB) as described (7). Four hundred crosses were set up to look for new insertion sites. In this study, only autosomal insertions were examined. Sixty-eight GAL4 insertion lines were obtained and balanced using standard genetic methods. Each of these was crossed to either Bg41-2 or Bg4-2-46 upstream activating sequence (UAS)–lacZ reporter lines, and β-galactosidase staining patterns were determined in embryos, imaginal discs, and ovaries. Out of 68 lines, 54 lines produced a detectable staining pattern. Lines that produced an interesting GAL4 expression pattern based on β-galactosidase staining were then crossed to a UAS–GFP line. The UAS–GFP line used in these experiments, GFPB1, contains a homozygous viable insertion on chromosome 3.

UAS–GFP Construct. The GFP coding region was isolated from plasmid TU#65, which contains the GFP cDNA in pBS(+) (Stratagene) as a *Kpn* I–*Eco*RI fragment and subcloned into pUAST. pUAST is a *P*-element vector based on pCaSpeR3 containing five optimal GAL4 binding sequences followed by a multiple cloning site (7).

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Abbreviations: GFP, green fluorescent protein; UAS, upstream activating sequence; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside.

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Transformations. Transgenic flies carrying the UAS–GFP construct were generated by injecting pUAS–GFP DNA at a concentration of 400 μg/ml with the helper plasmid pπ25.7wc at a concentration of 100 μg/ml into embryos of the w^1 strain (8, 9) using standard methods (10). A total of eight different lines were generated with the UAS–GFP insertion on X chromosome, chromosome 2, or chromosome 3.

Detection of *lacZ* by Antibody Immunocytochemistry and 5-Bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) Staining. The procedures used to collect embryos, remove the vitelline membrane, and stain whole-mount embryos with antibodies are described (11). The primary antibody, an IgG fraction rabbit anti-β-galactosidase from Cappel, was used at a concentration of 1:4000. The secondary antibody, a horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad), was used at 1:200. Imaginal discs were dissected in chilled phosphate-buffered saline (PBS) and fixed for 20 min in 0.75% glutaraldehyde. After being washed once with PBS/0.1% Triton X-100, cells were stained as described (12) and mounted in 70% glycerol. Ovaries were dissected and fixed in 1% glutaraldehyde for 20 min. Fixative was removed, and the ovaries were stained as described (13). After being washed in PBS/0.1% Triton X-100, they were mounted in 98% glycerol. Photographs of β-galactosidase histochemistry and immunocytochemistry were taken on a Nikon Optiphot 2 microscope with Nomarski optics, using a Nikon FX-35 camera.

Visualization of GFP Using Confocal Microscopy. Confocal images of GFP expression were taken on either a Leica DM IRB inverted laser confocal microscope using a standard fluorescein isothiocyanate filter providing excitation at 490 nm and emission at 527 nm or a Bio-Rad model MRC600 using a standard fluorescein isothiocyanate filter on a Nikon Optiphot 2 microscope. In all cases, image files were processed using a computer-based graphic system (Corel 4.0) where they were arranged and annotated. Images acquired of imaginal discs, the larval nervous system, and developing egg chambers were not further processed. All other images were processed to adjust the brightness and contrast of the image using Corel PHOTOPAINT. Imaginal discs from F_1 larvae resulting from a GAL4 line/UAS–GFP cross were dissected in distilled water and mounted immediately in 70% (vol/vol) glycerol/30% 0.1 M Tris (pH = 9). Larval central nervous systems were dissected in Schneiders medium according to standard procedures (14). F_1 embryos were dechorionated with 3% sodium hypochlorite, rinsed with distilled water, and mounted in 70% glycerol/30% 0.1 M Tris (pH = 9.0). Ovaries were dissected and mounted in PBS from 2-day-old F_1 virgin females. No fixatives were used in any of these preparations. Images of GFP expression in developing ovaries were derived as follows. Ovaries were dissected in Schneider's medium/10% fetal calf serum. Stage-8 egg chambers were dissected out of the epithelial sheath overlaying ovarioles and transferred to a microscope slide with medium. An artificial well was created on the slide using stacked slips of paper covered with vacuum grease to hold enough medium to bathe the sample and to support a coverslip. A *z*-series of confocal images was then obtained every hour for a total of 4 hr.

RESULTS

To determine whether GFP could be used as a vital marker/reporter in a variety of tissues during *Drosophila* development we generated transgenic lines containing the GFP cDNA under the transcriptional control of the yeast UAS. Three independent lines were tested by crossing them to several GAL4 enhancer trap lines that we had generated according to the protocol outlined in Brand and Perrimon (7). No apparent difference was observed in the ability to detect GFP from any of the three lines tested. The results presented here were obtained with the GFP-B1 line, which is a homozygous viable insertion of the UAS–GFP transgene on chromosome 3.

Expression of GFP was first examined by crossing the UAS–GFP-B1 line to three GAL4 enhancer trap lines that are expressed within adult ovaries. In all cases, appropriate expression of the UAS–GFP transgene was confirmed by comparing the results obtained with histochemical results of parallel crosses of the GAL4 lines with a UAS–lacZ line (Fig. 1 *A, C,* and *E*). The GAL4 lines used in these experiments targeted expression of GFP to posterior follicle cells (Fig. 1*B*), stalk cells (Fig. 1*D*), and nurse-cell-associated follicle cells (Fig. 1*F*). Expression of GFP could be detected in both freshly dissected ovaries and in fixed tissue (data not shown). By using the GFP marker in combination with confocal microscopy, we obtained greater resolution of the expression pattern than observed using X-Gal staining. The apparent reduction in the number of cells that express GFP in the posterior and nurse-cell-associated follicle cells is due to the optical sectioning of the confocal microscope (Fig. 1 *B* and *F*). Thus, GFP can be expressed and detected in ovaries not only as a fusion protein (6) but as a reporter gene as well. As previously noted (6), we could also detect minimal levels of autofluorescence within late egg chambers. However, using a barrier filter with a wavelength cut-off of 580 nm, we could distinguish between GFP and autofluorescence: GFP emits light maximally at 509 nm and is not detectable under these conditions, whereas autofluorescence can still be observed.

We also examined whether GFP could be detected in larval tissues by crossing the UAS–GFP-B1 line to several GAL4

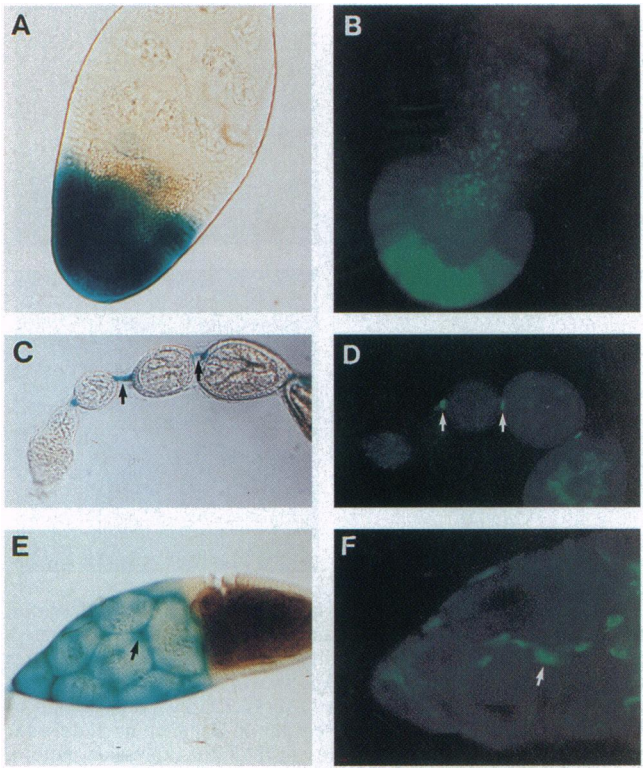

FIG. 1. Detection of GFP during oogenesis. Three GAL4 enhancer trap lines were used to detect expression of GFP during oogenesis. Expression of GFP was confirmed by crossing each GAL4 line to a UAS–lacZ line and staining ovaries with X-Gal. *A, C,* and *E* represent X-Gal staining; *B, D,* and *F* represent the same GAL4 lines examined for GFP expression. (*A* and *B*) GAL4 line A62, which directs expression of β-galactosidase and GFP in posterior follicle cells. (*C* and *D*) GAL4 line A39, which directs expression of β-galactosidase and GFP to stalk cells (see arrows). (*E* and *F*) GAL4 line A90, which directs expression of β-galactosidase and GFP to nurse-cell-associated follicle cells (see arrows). (*A–D*, ×94; *E*, ×100.)

enhancer trap lines that target GFP expression to specific cells in imaginal discs and the larval nervous system (Fig. 2). Illustrated are examples where GFP expression is targeted to the optic lobe (Fig. 2*B*), the eye disc and optic lobe (Fig. 2*D*), and a wing imaginal disc (Fig. 2*F*). Interestingly, when GFP was expressed in optic lobe neurons, the protein was not restricted to cell bodies within the larval nervous system but could also be detected in their processes. As seen in Fig. 2*D*, GFP clearly marked photoreceptor axons as they projected from the eye disc to innervate the optic lobe.

To further examine whether GFP could be used to identify nerve terminals, we used GAL4 enhancer trap lines to target expression of GFP to motoneurons within the larval nervous system (Fig. 3). These experiments clearly demonstrated that GFP could be detected both in neuronal cell bodies and in the processes immediately extending from the cell bodies (Fig. 3*A*). Similar to that observed in *C. elegans* (5), GFP could also be detected within nerve terminals at the point where they innervated specific muscles. For example, in a nerve terminal that innervates muscle 12 of the larval abdomen (Fig. 3*B*), GFP clearly outlines both the preterminal region and synaptic boutons (Fig. 3*B*). The only significant photobleaching observed was within the nerve terminals and seen only after prolonged exposure to the laser beam. However, fluorescence was recovered after a brief rest period in the absence of the laser beam. In the larval tissues examined, no autofluorescence was observed, and it was necessary to artificially increase the background by adjusting the baseline fluorescence using the black level control. This adjustment increased the total brightness of the image by a constant and allowed us to visualize the underlying structures for photography.

These experiments clearly demonstrate that GFP can be used to detect gene expression in a variety of freshly dissected tissues in *Drosophila* without any requirement for fixation or additional substrates. To determine whether GFP can also be used in intact living animals we examined GFP expression in embryos and larva (Fig. 4). Embryos were dechorionated, mounted in halocarbon oil on a glass slide, and viewed by confocal microscopy. Exposure of the embryo to the laser beam for the short periods of time required to obtain an image

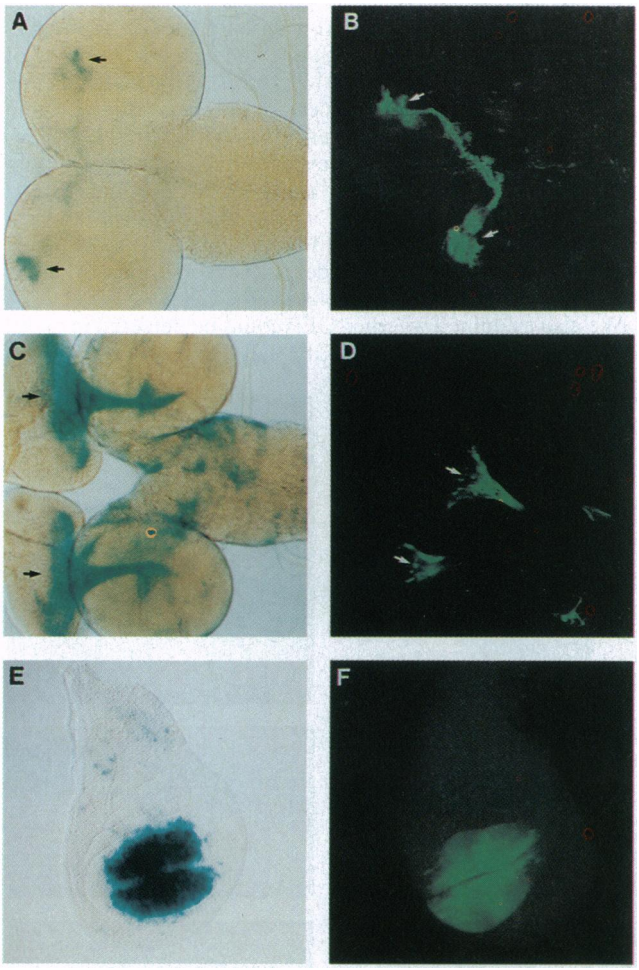

FIG. 2. Detection of GFP in larval imaginal discs and central nervous system. Three GAL4 enhancer trap lines were used to detect expression of GFP in larval imaginal discs and the central nervous system. To confirm that GFP was expressed in the appropriate pattern, each GAL4 line was crossed to a UAS–lacZ line and examined by X-Gal staining. *A*, *C*, and *E* represent the X-Gal staining pattern for each GAL4 line; *B*, *D*, and *F* represent the GFP expression pattern. (*A* and *B*) GAL4 line A95, which directs expression of β-galactosidase and GFP to neuronal cell bodies within the optic lobe and to their processes that extend from one lobe to the other (see arrows). The optic lobes are located at left and the ventral ganglia are located at right. In this preparation, the eye discs have been removed. (*C* and *D*) GAL4 line B41, which directs expression of β-galactosidase and GFP to photoreceptor neurons within the eye disc and to their processes, which innervate deep within the optic lobe. Arrows indicate location of the photoreceptor cells within the eye disc. (*E* and *F*) GAL4 line C5, which directs expression of β-galactosidase and GFP to the region of the wing imaginal disc, which will give rise to the wing blade. (*A–F*, ×110.)

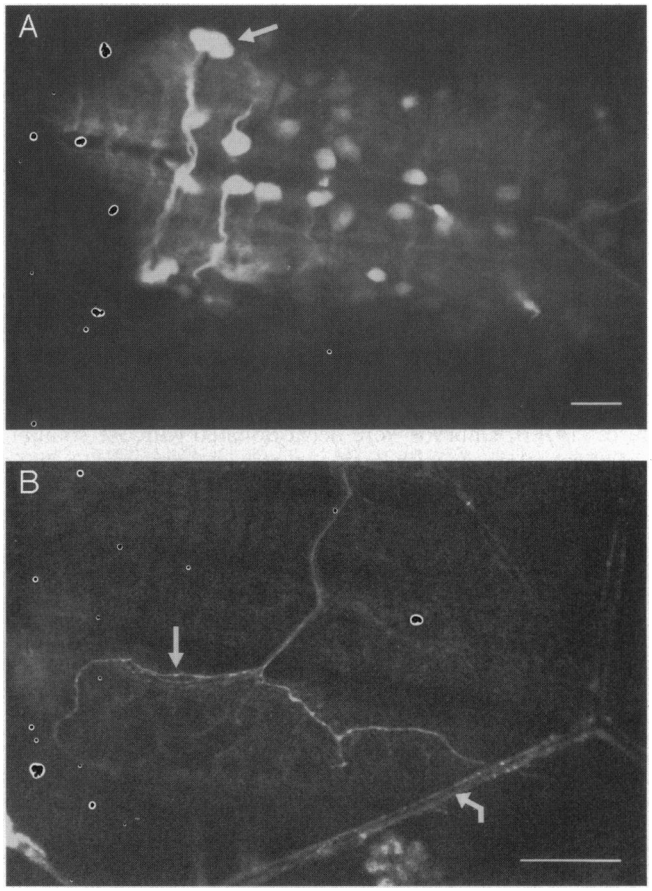

FIG. 3. Detection of GFP in motoneuron cell bodies and nerve terminals. The GAL4 enhancer trap line D42 was used to target expression of GFP to motoneurons within a living third-instar larvae. (*A*) Expression of GFP can be detected within motoneuron cell bodies and in the processes that immediately extend from them. The arrow points to a specific motoneuron within the larval CNS. (*B*) Expression of GFP can also be detected at the nerve terminal and within synaptic boutons (straight arrow). Autofluorescence can also be detected within the trachae (bent arrow). (Bar = 50 μm.)

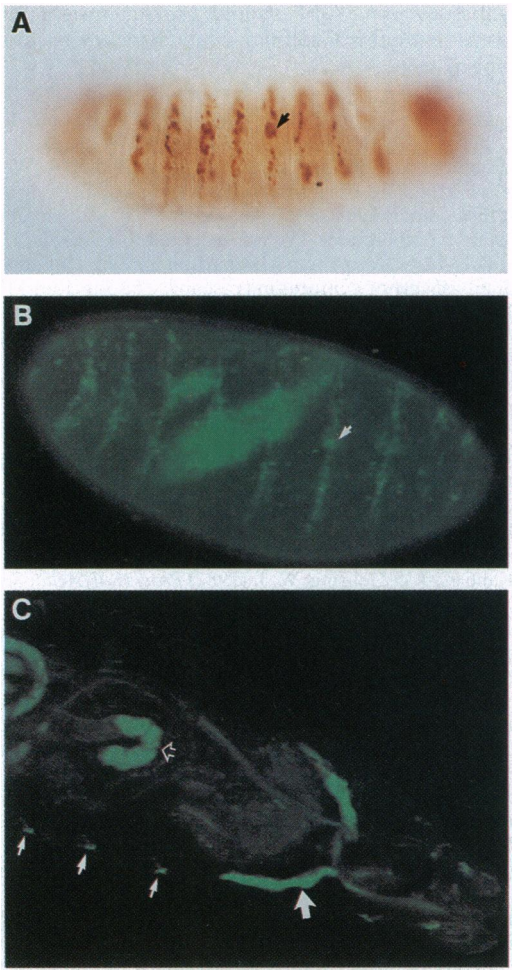

FIG. 4. Detection of GFP in living embryos and larvae. Expression of GFP can also be detected in embryos or larvae without any prior dissection or fixation. (*A*) Anti-β-galactosidase staining of embryos expressing β-galactosidase driven from the GAL4 line C41. Expression can be detected in most, but not all, of the cells which comprise the peripheral nervous system of the embryo. The arrow points to a group of cells within the lateral cluster of the peripheral nervous system. (*B*) Expression of GFP driven by the GAL4 line C41 can be detected within the peripheral nervous system of live embryos. (*C*) Expression of GFP driven by the GAL4 line C38 can be detected within the larval salivary glands (large filled arrow) and pair of cells along the body wall within each segment (small filled arrows). Autofluorescence from the gut is indicated by an open arrow. (*A–C*, ×55.)

(<1 min) did not alter its viability, and embryos were observed to hatch into larvae (data not shown). Expression of GFP is shown for one GAL4 line, C41, which is expressed in much of the embryonic peripheral nervous system (Fig. 4*B*). Appropriate expression of GFP was confirmed by immunostaining embryos with an anti-β-galactosidase antibody (Fig. 4*A*). While all of the peripheral neurons cannot be observed in a single confocal optical section, the overall pattern is maintained. Similar observations can also be made in intact larvae. Fig. 4*C* shows a GAL4 enhancer trap line that targets GFP to both salivary glands and pairs of cells that are distributed segmentally along the larval body wall. In larvae, as in embryos, development was not affected by exposure to the laser beam. In contrast to larval imaginal discs and nervous system, whole embryos and larvae have significant levels of autofluorescence due to the yolk and gut, respectively. These levels, however, could be resolved by using appropriate barrier filters as described above.

To determine whether GFP could be used to detect dynamic changes in gene expression within living tissues in real time, we

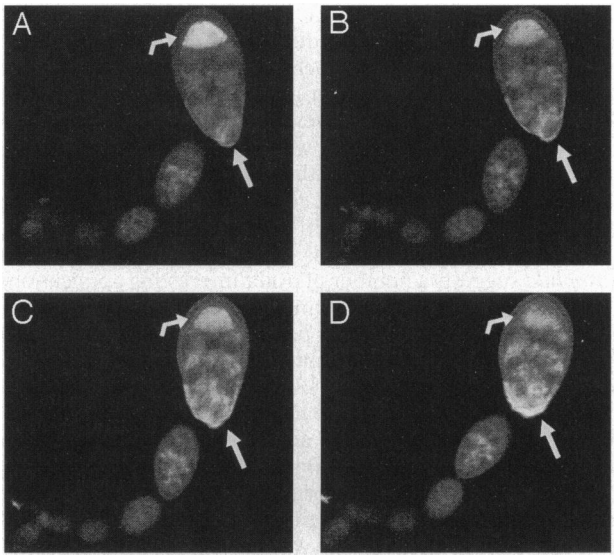

FIG. 5. Changes in GFP expression can be detected in cultured egg chambers. Expression of GFP was monitored in cultured stage-8 egg chambers during a 4-hr period. A *z*-series of confocal images was obtained every hour for the entire 4-hr period. Images represent a particular focal plane from each *z*-series. Expression of GFP in egg chambers is directed by GAL4 line A90, which targets expression to nurse-cell-associated follicle cells. (*A*) Detection of GFP after 1 hr in culture. Little to no expression of GFP can be detected within follicle cells (straight arrow). Low levels of autofluorescence, however, can be observed within the oocyte (bent arrow). (*B*) GFP expression begins to be detected after 2 hr in culture in follicle cells at the anterior end of the stage-8 egg chamber (straight arrow). In contrast, the level of autofluorescence from the oocyte decreases (bent arrow), and no changes are observed in earlier egg chambers. (*C*) After 3 hr, levels of GFP expression within nurse-cell-associated follicle cells increase (straight arrow). (*D*) After 4 hr, GFP expression can be detected in all nurse-cell-associated follicle cells and is particularly high in follicle cells at the anterior tip of the egg chamber (straight arrow). No changes in fluorescence are detected in earlier egg chambers, and the autofluorescence in the oocyte remained low (bent arrow). (*A–D*, ×90.)

performed time-lapse confocal microscopy of developing egg chambers (Fig. 5). These experiments were done by using a GAL4 enhancer trap line, A90, which targets expression of UAS–GFP to nurse-cell-associated follicle cells (Fig. 1*C*). Egg chambers that were dissected and cultured in Schneiders medium over a period of 4 hr are shown. Initially, no GFP could be detected within stage-8 egg chambers (Fig. 5*A*). However, by 1 hr, GFP expression was observed at the anterior end of the stage-8 egg chamber, and this expression increased steadily over time (Fig. 5 *B–D*). Expression of GFP is specific to nurse-cell-associated follicle cells and restricted to stage-8 egg chambers. No GFP could be detected in stage-2 to -7 egg chambers. These results clearly show that GFP can be used as a reporter to monitor activation of gene expression in living tissue over time. Whether GFP can also be used to monitor cessation of gene expression remains to be determined and will depend on the stability of GFP in various cell types during development.

DISCUSSION

The ability to study development as it occurs within an organism relies on the availability of techniques that can detect changes in gene expression within specific cells or tissues during cell movements and migrations. We have used the GFP from the jellyfish, *A. victoria*, as a viable marker in *Drosophila* to observe such changes within living tissues. GFP was expressed as a nonfused protein under the transcriptional control of a yeast UAS and targeted to specific cell types during

development by crossing to a variety of GAL4-expressing enhancer trap lines. These studies clearly show that GFP can be used as a reporter gene, much like bacterial *lacZ*, to detect expression of specific genes in a variety of cell types during *Drosophila* development. However, in contrast to *lacZ*, GFP can be visualized in live tissue without fixation or addition of specific substrates and often without any dissection. This result permits the monitoring of gene expression within a living organism over time.

The ability to use GFP as a vital marker/reporter in *Drosophila* suggests a number of other interesting applications. For example, development of an enhancer trap vector system based on GFP suggests the possibility of bulk screening, whereby specific expression patterns could be detected in embryos in the F_1 generation. Because GFP can function as a reporter gene, it should be able to replace *lacZ* in other assays, such as promoter mapping. In addition, it may be possible to use GFP to sort pure populations of live cells using a fluorescence-activated cell sorter as originally described by Krasnow *et al.* (15) using a fluorogenic β-galactosidase substrate. GFP could also be recombined onto various balancer chromosomes to allow for rapid identification of embryos/larvae containing the specific balancer, much like *lacZ*-marked balancers that are currently available. GFP balancers would permit identification and selection of homozygous mutants based on the absence of GFP expression. *P*-element vectors could also be generated using GFP as a reporter gene and used to characterize various mutant phenotypes arising from *P*-element insertional mutagenesis. Because GFP is expressed not only in cell bodies but also in processes, this may be particularly useful in identifying axon guidance or pathfinding mutants. Until recently, these mutants were difficult to identify, as β-galactosidase fails to readily diffuse into axons. Alternative approaches in which β-galactosidase is expressed as a kinesin–lacZ (16) or Tau–lacZ fusion protein (17) are more efficient at detecting axonal processes but still require fixation and the addition of specific substrates or antibodies to detect expression, which only provides a static image of the axonal process. The expression of GFP in nerve terminals may also be useful for studying synaptogenesis. For example, GAL4 lines that target expression of GFP to photoreceptor axons could be used to examine their ability to form appropriate synapses within the optic lobe. Expression of GFP in nerve terminals could also be used to identify specific neurons and/or synaptic boutons, which could then be analyzed electrophysiologically.

Finally, we have shown that GFP can be used to detect changes in gene expression in living tissue. This result suggests that GFP could be used in fate mapping or lineage analysis experiments. GFP could also be used to monitor changes in cell migrations or cell shape such as occur during germ-band extension (18) and the migration of pole cells (19), follicle cells (13), and tracheal cells (20, 21) during development. The identification of mutations that affect the pattern of specific cell migrations combined with the ability to visualize the cells in living tissues using GFP should provide insight into the mechanisms that control cell movements as they occur within the organism.

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